PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Richard J. Budhu, et al.		
Serial No.:	10/505,257	Case: 21044P	Art Unit: 1626
Filed:	August 20, 2004		
For:	AMINOALKYLPHOSPHANATES AND RELATED COMPOUNDS AS EDG RECEPTOR AGONISTS		Examiner: Chu, Yong Liang

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF SUZANNE M. MANDALA

Sir:

I, Suzanne M. Mandala, hereby declare the following:

1. I received a B.A. in Biology from Swarthmore College in 1979;

2. I received a Ph.D. in Biology from the University of California, Santa Cruz in 1985;

3. I have been employed by Merck & Co., Inc. beginning in 1989 and currently hold the title of Director in the Department of Immunology & Rheumatology;

5. I have reviewed United States Patent Application No. 10/505,257 having a PCT filing date of February 27, 2003 and published on April 27, 2006 as United States Patent Application Publication No. 2006/0089334;

6. In my laboratory, I have supervised the use of the protocols described on pages 65 to 67 of the captioned patent application and paragraphs 208 to 213 of the United States patent application publication described above under the headings **Ligand Binding to Edg/S1P Receptor Assay** and **^{35}S-GTPγS Binding Assay**. Copies of these protocols are provided at the end of this Declaration;

7. I supervised the testing of Examples 1 to 37 described in the captioned patent application in the **Ligand Binding to Edg/S1P Receptor Assay**. I also supervised the testing of Examples 2, 3, 5, 8, 10, 12, 13, 14, 15 and 26 described in the captioned patent application in the **^{35}S-GTPγS Binding Assay**. The results are shown in the following table. The provided values are the mean of at least two replicates.

EXAMPLE	S1P1 IC_{50} (nM)	S1P1 EC_{50} (nM)
1	0.8	
2	20	8.7
3	3.3	2.9
4	14	
5	620	40
6	570	
7	1200	
8	620	91
9	510	
10	360	130
11	120	
12	1.3	1.0
13	3.5	3.2
14	26	20
15	13	9.7
16	1.8	
17	20	
18	1.9	
19	0.7	
20	3900	
21	1100	
22	130	
23	1100	
24	420	
25	1.0	
26	0.6	0.3

EXAMPLE	S1P1 IC_{50} (nM)	S1P1 EC_{50} (nM)
27	2000	
28	460	
29	53	
30	180	
31	480	
32	410	
33	37	
34	94	
35	19	
36	1000	
37	170	

8. I hereby declare that all statements made herein of my own knowledge are true and that all statement made of information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted,

Suzanne Mandala

Date: April 26, 2007

Suzanne M. Mandala

Ligand Binding to Edg/S1P Receptors Assay

^{33}P-sphingosine-1-phosphate was synthesized enzymatically from γ^{33}P-ATP and sphingosine using a crude yeast extract with sphingosine kinase activity in a reaction mix containing 50 mM KH_2PO_4, 1 mM mercaptoethanol, 1 mM Na_3VO_4, 25 mM KF, 2 mM semicarbazide, 1 mM Na_2EDTA, 5 mM $MgCl_2$, 50 mM sphingosine, 0.1% TritonX-114, and 1 mCi γ^{33}P-ATP (NEN; specific activity 3000 Ci/mmol). Reaction products were extracted with butanol and ^{33}P-sphingosine-1-phosphate was purified by HPLC.

Cells expressing EDG/S1P receptors were harvested with enzyme-free dissociation solution (Specialty Media, Lavallette, NJ). They were washed once in cold PBS and suspended in binding assay buffer consisting of 50 mM HEPES-Na, pH 7.5, 5mM $MgCl_2$, 1mM $CaCl_2$, and 0.5% fatty acid-free BSA. ^{33}P-sphingosine-1-phosphate was sonicated with 0.1 nM sphingosine-1-phosphate in binding assay buffer; 100 µl of the ligand mixture was added to 100 µl cells (1 x 10^6 cells/ml) in a 96 well microtiter dish. Binding was performed for 60 min at room temperature with gentle mixing. Cells were then collected onto GF/B filter plates with a Packard Filtermate Universal Harvester. After drying the filter plates for 30 min, 40 µl of Microscint 20 was added to each well and binding was measured on a Wallac Microbeta Scintillation Counter. Non-specific binding was defined as the amount of radioactivity remaining in the presence of 0.5 µM cold sphingosine-1-phosphate.

Alternatively, ligand binding assays were performed on membranes prepared from cells expressing Edg/S1P receptors. Cells were harvested with enzyme-free dissociation solution and washed once in cold PBS. Cells were disrupted by homogenization in ice cold 20 mM HEPES pH 7.4, 10 mM EDTA using a Kinematica polytron (setting 5, for 10 seconds). Homogenates were centrifuged at 48,000 x g for 15 min at 4°C and the pellet was suspended in 20 mM HEPES pH 7.4, 0.1 mM EDTA. Following a second centrifugation, the final pellet was suspended in 20 mM HEPES pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$. Ligand binding assays were performed as described above, using 0.5 to 2 µg of membrane protein.

Agonists and antagonists of Edg/S1P receptors can be identified in the ^{33}P-sphingosine-1-phosphate binding assay. Compounds diluted in DMSO, methanol, or other solvent, were mixed with probe containing ^{33}P-sphingosine-1-phosphate and binding assay buffer in microtiter dishes. Membranes prepared from cells expressing

Edg/S1P receptors were added, and binding to ^{33}P-sphingosine-1-phosphate was performed as described. Determination of the amount of binding in the presence of varying concentrations of compound and analysis of the data by non-linear regression software such as MRLCalc (Merck Research Laboratories) or PRISM (GraphPad Software) was used to measure the affinity of compounds for the receptor. Selectivity of compounds for Edg/S1P receptors was determined by measuring the level of ^{33}P-sphingosine-1-phosphate binding in the presence of the compound using membranes prepared from cells transfected with each respective receptor ($S1P_1$/Edg1, $S1P_3$/Edg3, $S1P_2$/Edg5, $S1P_4$/Edg6, $S1P_5$/Edg8).

^{35}S-GTPγS Binding Assay

Functional coupling of S1P/Edg receptors to G proteins was measured in a ^{35}S-GTPγS binding assay. Membranes prepared as described in the Ligand Binding to Edg/S1P Receptors Assay (1-10 μg of membrane protein) were incubated in a 200 μl volume containing 20 mM HEPES pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 5 μM GDP, 0.1% fatty acid-free BSA (Sigma, catalog A8806), various concentrations of sphingosine-1-phosphate, and 125 pM ^{35}S-GTPγS (NEN; specific activity 1250 Ci/mmol) in 96 well microtiter dishes. Binding was performed for 1 hour at room temperature with gentle mixing, and terminated by harvesting the membranes onto GF/B filter plates with a Packard Filtermate Universal Harvester. After drying the filter plates for 30 min, 40 μl of Microscint 20 was added to each well and binding was measured on a Wallac Microbeta Scintillation Counter.

Agonists and antagonists of S1P/Edg receptors can be discriminated in the ^{35}S-GTPγS binding assay. Compounds diluted in DMSO, methanol, or other solvent, were added to microtiter dishes to provide final assay concentrations of 0.01 nM to 10 μM. Membranes prepared from cells expressing S1P/Edg receptors were added, and binding to ^{35}S-GTPγS was performed as described. When assayed in the absence of the natural ligand or other known agonist, compounds that stimulate ^{35}S-GTPγS binding above the endogenous level were considered agonists, while compounds that inhibit the endogenous level of ^{35}S-GTPγS binding were considered inverse agonists. Antagonists were detected in a ^{35}S-GTPγS binding assay in the presence of a sub-maximal level of natural ligand or known S1P/Edg receptor agonist, where the compounds reduced the level of ^{35}S-GTPγS binding. Determination of the amount of binding in the presence of

varying concentrations of compound was used to measure the potency of compounds as agonists, inverse agonists, or antagonists of S1P/Edg receptors. To evaluate agonists, percent stimulation over basal was calculated as binding in the presence of compound divided by binding in the absence of ligand, multiplied by 100. Dose response curves were plotted using a non-linear regression curve fitting program MRLCalc (Merck Research Laboratories), and EC_{50} values were defined to be the concentration of agonist required to give 50% of its own maximal stimulation. Selectivity of compounds for S1P/Edg receptors was determined by measuring the level of ^{35}S-GTPγS binding in the presence of compound using membranes prepared from cells transfected with each respective receptor.